Germinal center reaction

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The germinal center reaction is one critical outcome of helper T-cell–dependent antigen-specific B-cell responses. Germinal center reactions are the culmination of an orchestrated series of intercellular information exchanges discussed here as the serial synapsis model of adaptive immunity. The main purpose of the germinal center reaction is the development of B-cell memory through iterative cycles of somatic antigen receptor diversification and the selection of B cells with receptors of best fit. Recent studies provide insight into the regulation of these complex processes *in vivo* with new information on the cellular organization of the memory B-cell compartment. Curr Opin Hematol 2001, 8:52–59

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Abbreviations:

GC	germinal center
TH	helper T
MHC	major histocompatibility complex
DC	Dendritic cells
BLC	B lymphocyte chemoattractant
ELC	Epstein-Barr virus-induced molecule 1 ligand
SLC	secondary lymphoid chemokine
CXCR	CXC chemokine receptor
ICOS	inducible co-stimulator
BAFF	B cell activating factor belonging to the TNF family
LT β	lymphotoxin β
TNF	tumor necrosis factor
GCDC	germ center dendritic cells
AID	activation-induced cytidine deaminase

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The germinal center (GC) is a dynamic microenvironment that emerges in the follicular regions (B-cell zones) of secondary lymphoid organs in response to antigenic priming. These structures originate from the extensive local expansion of antigen-primed B-cell clones within B-cell follicles (primary follicles) that eventually reach ~10^4 cells per GC. Cognate T-cell help is required for normal development of the GC reaction, and there is clear evidence for antigen-specific helper T (Th) cells within each GC microenvironment. Follicular dendritic cells (FDC) also play a major role in the GC cycle of events that gives rise to affinity-matured long-lived memory B cells. The authors focus on recent studies of 1) induction of the GC reaction, 2) the GC reaction itself and 3) the post-GC phase of the immune response.

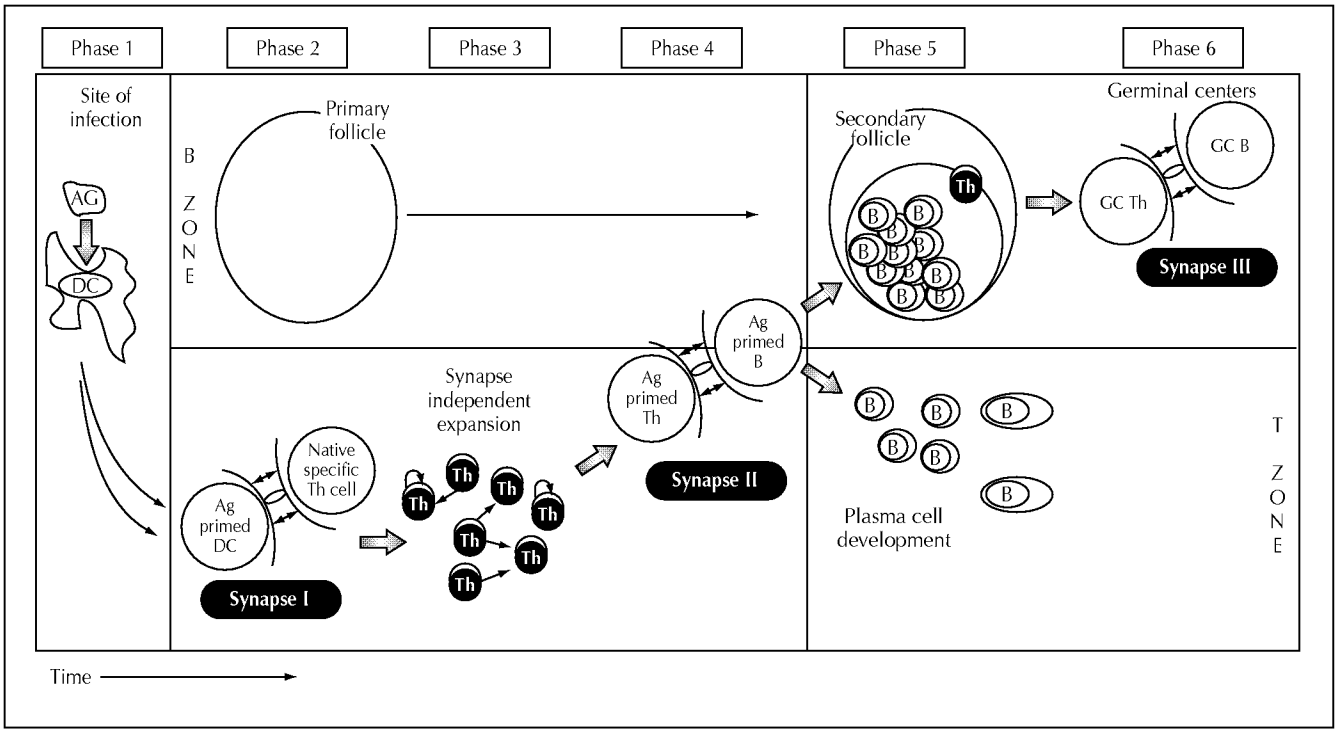
Induction of the germinal center reaction

The recognition of processed antigenic peptides in major histocompatibility complex (MHC) molecules is central to the regulation of adaptive immunity by antigen-specific Th cells. Seminal work by Kupfer and colleagues [1,2] characterized the molecular organization of cognate interactions between Th cells and antigen-presenting cells (APC). These interactions consist of a central cluster of T-cell receptor–peptide/MHC molecules surrounded by clusters of complementary adhesion molecule interactions [1,2]. Dustin and colleagues have quantified the dynamics of these molecular interactions using both T-cell lines and proteins in lipid bilayers [3••,4••]. This clustering phenomenon is now broadly referred to as the formation of an immunologic synapse. The actin cytoskeleton plays a critical role in synapse formation [5,6], enabling the translocation of major co-stimulatory molecules (such as B7-CD28 and ICAM-1-LFA-1) to the cellular interface [7•]. Cytoskeletal rearrangement may also recruit membrane microdomains rich in glycolipids and signaling molecules into the synapse, encouraging efficient downstream activity [5]. Further, there is evidence of suboptimal recruitment of signaling intermediates into the Th/APC immune synapse associated with immune deficits in aging [8,9]. Hence, immune synapse formation appears to be the organizing principle underlying cognate cellular interactions *in vivo*.

Serial synapsis model

In Figure 1, we present Th-cell–dependent B-cell responses as a series of six overlapping phases of synapse-dependent and synapse-independent develop-

Figure 1: The serial synapsis model



The six phases depicted in this model are used to broadly outline the synapse-dependent and synapse-independent phases of a developing primary immune response. Phase 1 depicts the initiation of DC activation at the site of antigen insult. The antigen-primed DC localizes to the T-cell zones of secondary lymphoid organs to recruit naive but antigen-specific Th cells to form the first immune synapse (phase 2: synapse I). Clonal expansion of the antigen-specific Th cells ensues in the T-cell zone in a synapse-independent manner (phase 3). At this point autocrine and paracrine influences help to propagate patterns of Th-cell development that were initiated at first contact. There is significant migration of these antigen-primed Th cells to the follicular borders of these organs. The next stage involves cognate delivery of T-cell help to antigen-primed B cells and the less–well-characterized signaling to the Th cells by antigen-primed B cells (phase 4: synapse II). The outcome of this information exchange is clonal expansion of antigen-specific B cells and their subsequent differentiation into short-lived plasma cells in the T-cell zones or alternatively, secondary follicle formation in the B-cell zones (phase 5: synapse-independent). Antigen-specific Th cells also migrate to the follicular regions during this phase of the response. The last phase involves polarization of the secondary follicle to form the germinal center reaction (depicted in more detail in Fig 2) (phase 6: synapse III). The main function of the GC reaction is B-cell affinity maturation. Delivery of T-cell help in this microenvironment would also require formation of an immune synapse between GC Th cells and GC B cells.

ment. This model attempts to integrate our understanding of known cellular interactions, their timing, and microenvironmental localization and includes a requirement for synapse formation in the cognate cellular exchange of information. This complex adaptive response to antigen culminates in the formation of the germinal center (GC) reaction (phase 6) as the site of memory B-cell development.

Phase 1 signifies the initial activation of resident APC at the site of antigen entry through nonspecific means of antigen uptake. Dendritic cells (DC), as the most efficient APC, activate in response to antigen and local inflammatory signals and migrate to the T-cell zones of draining lymph nodes. In the T-cell zones, primed DCs recruit naive antigen-specific Th cells and initiate the first cognate cellular interactions (phase 2: synapse I). It is generally thought that all naive Th cells are equally able to differentiate into a spectrum of effector Th cells as pluripotent antigen-specific precursors. However, the quality of the TCR-peptide/MHC interaction can also clearly influence functional commitment of Th cells [10••]. Hence, the available TCR repertoire can influence the outcome of an immune response [11,12••]. This initial stage of antigen-specific Th-cell development is heavily influenced by the DC expression pattern of cytokines (such as IL-12 and IL-6) and costimulatory molecules (such as CD80 and CD86). The antigen-primed Th cells can then deliver signals to the DC by way of cell contact (such as CD40L) and perhaps also immediate early cytokine production (such as TNF-α). Thus, intercellular synapsis encourages efficient local exchange of molecular information that remains antigen-specific.

The next phase involves synapse-independent clonal expansion of antigen-specific Th cells (phase 3: synapse independent). At this stage, developmental programs initiated during the first synapse can be consolidated and propagated through selective cellular expansion.

Selection for Th cells with preferred TCR occurs very rapidly (days 3–5 after priming) and is associated with extensive clonal expansion in the T-cell zones [13••]. Subdominant clonotypes selected in this phase of the response express distinct cell fates (such as GC versus non-GC entry)[14•]. Autocrine and paracrine influences of cytokines may also play a major role in shaping the mix of Th-cell functions within the responsive population during this phase of the response. Our recent studies on cytokine production in vivo clearly demonstrate a broad spectrum of functional outcomes associated with the Th-cell response to one dominant peptide epitope [15•]. This phase of synapse-independent development is also associated with the migration of antigen-specific Th cells toward the T/B follicular borders *in vivo* [16] to initiate the second critical synaptic interaction between antigen-primed Th cells and antigen-primed B cells.

A role for chemokines

The CXC chemokine receptor (CXCR) 5 is expressed on antigen-activated Th cells, and induces responsiveness to B lymphocyte chemoattractant (BLC) [17•]. BLC is critical for organizing primary B-cell follicles and FDC networks in the spleen and lymph nodes [18••]. Engagement of CD28 and upregulation of OX40, most likely phase 2 events, precede CXCR5 expression and the follicular migration of antigen-activated Th cells [19]. The increased response to BLC is accompanied by a decreased response to Epstein-Barr virus-induced molecule 1 ligand (ELC) and secondary lymphoid chemokine (SLC)[17•]. Both ELC and SLC are found predominantly in the T-cell zones and are ligands for CCR7. The expression of CCR7 is constitutive on resting Th cells and is required for their migration to peripheral LNs[20]. CCR7 is also needed for DC migration to LNs after initial activation[20]. Interestingly, memory Th cells in humans can be distinguished as two functionally distinct subsets based on CCR7 expression, migration capabilities, and rapid expression of cytokines [21••]. Although not as much is known about antigen-driven changes in the B-cell compartment, it is clear that changes in steady-state chemokine responsiveness [22] will accompany GC development *in vivo*.

Antigen-specific B cells must have bound, processed, and presented antigenic peptide-MHC class II complexes to initiate the formation of synapse II with antigen-primed Th cells (phase 4: synapse II). There is nearly a complete absence of GC formation in the absence of Cathepsin S, which blocks invariant chain degradation and delays peptide loading into class II MHC molecules [23]. Interfering with the processing of peptide in this model also markedly impairs the switch in antibody isotype to IgG2a and IgG3, but it does not affect IgE [23]. Whereas chemokine gradients increase the likelihood of Th/B-cell engagement, complimentary cell surface interactions are more likely to determine successful synapse II formation. In addition to CD28/CTLA4 and CD80/86 interactions, candidates for promoting Th/B-cell contact in synapse II formation include inducible co-stimulator (ICOS) on activated Th cells and B7-h on resting B cells [24••,25••,26]. B-cell activating factor belonging to the TNF family (BAFF), expressed on DC and activated Th cells, and its counter-receptor BAFF R on activated B cells may also play a role[27]. Finally, cytokine production by Th cells clearly influences B-cell development, but Nadler and colleagues demonstrate cytokine production by antigen-experienced human tonsillar B cells that may create positive feedback loops to influence Th-cell development [28]. IL-12 and IL-6 produced by B cells in cognate contact with Th1 or Th2 cells respectively could maintain or even amplify preferred Th-cell profiles *in vivo*. Thus, Th/B-cell synapsis also provides an opportunity for the focused exchange of molecular information.

Germinal center reaction

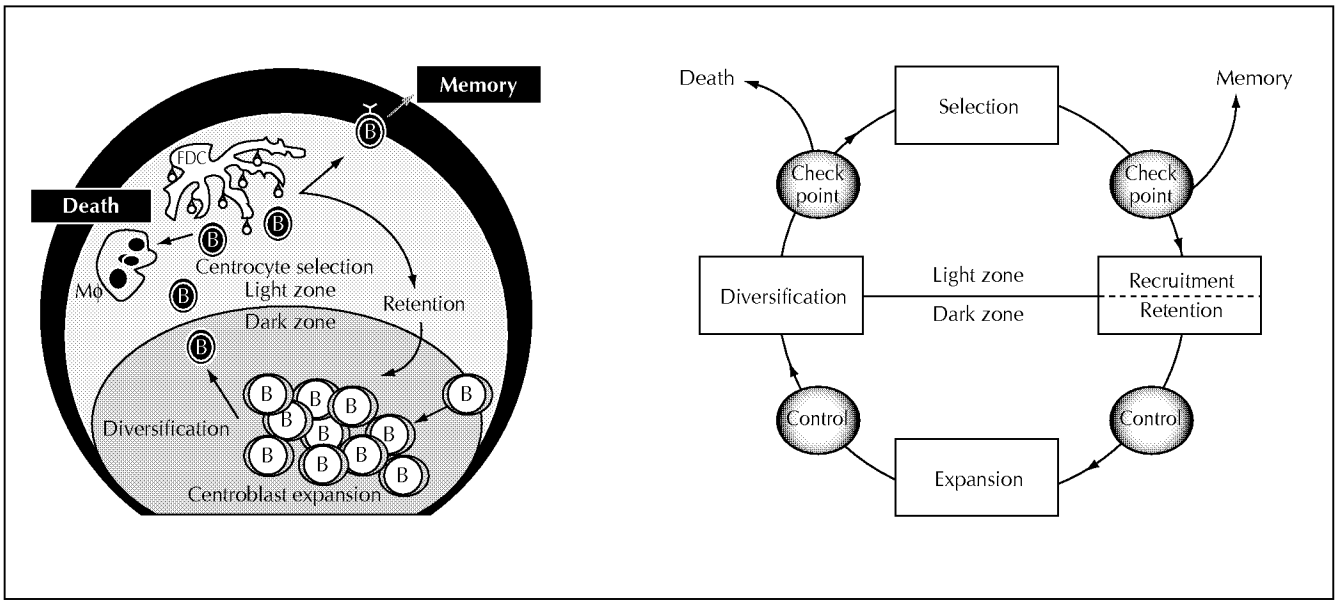
Plasma cell differentiation and secondary follicle formation

The predominant cellular outcome of synapse II is extensive clonal expansion of antigen-specific B cells (phase 5: synapse independent). Some of this B-cell expansion proceeds in the T-cell zones and gives rise to short-lived antibody-secreting plasma cells. Depending on the quality of the Th-cell interactions in synapse II, these plasma cells secrete IgM and downstream immunoglobulin isotypes. The remainder of the B-cell expansion proceeds in the B-cell zones within the primary follicles. Initial expansion occurs with a 6–8-hour doubling time and creates large IgD-negative regions within primary follicles (these regions are now referred to as secondary follicles). At approximately day 7–10 after initial priming, the secondary follicle polarizes into a T-cell zone-proximal region of rapidly-dividing B cells (centroblasts) and a region of quiescent non-cycling B cells (centrocytes) at the opposite pole. Once polarity is established, the dynamic microenvironment is called the germinal center reaction.

GC cycle

Figure 2 depicts the progress of B-cell development within the GC reaction and graphically outlines the accompanying molecular and cellular process (phase 5: synapse dependent)[29•]. Antigen-specific B cells recruited into the GC reaction rapidly expand (centroblasts) and then diversify their antigen receptors through somatic hypermutation and a process of receptor editing in the GC dark zone. The vast majority of these essentially random changes are deleterious to antigen binding and lead to death of the resulting centrocyte within the GC light zone. Some rare muta-

Figure 2: The germinal center cycle



This figure illustrates some of the cells involved in the antigen-specific GC developmental pathway (on the right) and the cellular and molecular processes typical of the GC reaction (on the left) that underpin the induction of B-cell memory. Antigen-specific B cells are recruited into the follicular regions within the B-cell zones of secondary LNs. Rapid clonal expansion describes the formation of the secondary follicle. The polarization of expanding centroblasts to the T proximal regions of the secondary follicle and the appearance of resting centrocytes apically defines the emergence of the GC reaction. Antigen-specific B cells diversify their BCR by somatic hypermutation and receptor editing and then 'test' their variant BCR for antigen binding in the light zone. Antigen is present in the GC as immune complexes deposited on the FDC networks of the light zone. Diminished binding for antigen leads to programmed cell death and rapid clearance of apoptotic B cells locally by tingle body macrophage (mΦ). Improved binding to antigen results in positive selection of the variant. There are two possible outcomes. The first is to return to the dark zone and resume the cycle of expansion, diversification, and selection. The second is to exit the GC cycle and thereby enter the memory B-cell compartment.

tions increase the cell's affinity for antigen binding, with the resulting centrocyte being positively selected to either remain within the GC and re-enter the cycle or exit into the long-lived memory B-cell pool. Next, we will review the recent literature pertaining to the four main cell types known to reside in the GC microenvironment (GC Th cells, FDC, GC DC and GC B cells) and the two broad activities (receptor diversification and cellular selection) that take place within the GC cycle.

Cells of the GC

The GC reaction is generally considered Th-cell dependent; however, in the presence of exaggerated numbers of specific B-cell precursors, even Th-independent responses can promote GC [30•]. Interfering with synapse I or II interactions (e.g., by blocking CD40:CD40L interactions) can clearly abort development of the GC reaction. However, the activity of antigen-specific Th cells within the GC has not been as clearly assessed. These GC Th cells have already been selected for preferred TCR [13••], with some antigen-specific clonotypes never reaching the GC reaction [14•]. There were earlier reports of TCR somatic hypermutation within GC Th cells but recent studies have found no evidence for this phenomenon, either in the total antigen-specific compartment [13••] or in Th cells selected from GC [31,32] (Mikszta JA, McHeyzer-Williams L, and McHeyzer-Williams M, submitted). If GC Th cells were to exert an influence on memory B-cell development, it would most likely involve cognate interactions and the formation of an immune synapse (phase 6: synapse III). Synapse III would most likely involve the exchange of information through complementary cell surface interactions and soluble factors that may critically impact on the progression of antigen-specific GC Th-cell development.

FDC appear to play an important role in affinity-based selection of memory B cells. Although GC can form without FDC networks, they regress rapidly with reduced affinity maturation [33]. Mature GC formation requires lymphotoxin (LT) β and tumor necrosis factor (TNF) production by B cells. FDC precursors need TNFRp55 and LTβR signals in the bone marrow for adequate maturation and subsequent GC formation [34•]. Analysis of FDC lines suggests that they provide potent proliferative signals for centroblasts [35] through novel molecules such as the 8D6 antigen [36]. IL13Ra expressed on FDC promotes IgG2a/G2b production through novel counter-receptors on GC B cells [37]. In these cases, GC Th cells are still impli-

cated in initiating or modulating GC B-cell differentiation. Germinal center DC (GCDC) have been implicated in regulating the GC Th cells, but they can now be shown to directly effect GC B-cell expansion and plasma cell differentiation through IL-12 production [38]. The GC B cells themselves appear to change over time *in vivo*, with BLA-1 expression providing one indicator for change [39•]. The polycomb group of homeotic gene regulators also changes with development of centroblasts from resting B cells and then returns to a quiescent expression pattern in centrocytes [40]. Finally, the repression of Ig enhancer function in OCA-B$^{-/-}$ mice appears to reduce both immunoglobulin isotype switch and GC formation *in vivo* [41]. Hence, at least four separate cell types play important roles in the progression of the GC reaction and development of B-cell memory.

B-cell antigen receptor diversification

The role of receptor editing in GC diversification has struck some controversy. Nussenzweig and colleagues have produced transgenic mice that co-express green fluorescent protein (GFP) and recombination-activating genes (*RAG*). Using this model, RAG expression appears continuous in some splenic B cells with no evidence for re-expression in the periphery [42•]. In a follow-up study, this group demonstrates the nonspecific (adjuvant-induced) accumulation of GFP$^+$ immature B cells in the spleen but no GC localization of *RAG* expression [43•]. Alt and colleagues use a GFP knock-in model that indicates loss of *RAG* expression in peripheral B cells and similar migration of GFP$^+$ immature cells late after immunization [44•]. *RAG* expression could be reinduced in mature splenic B cells with Ig cross-linking only in the presence of BM stromal cells. In this latter study, GL7 expression was used to demonstrate GC association of the GFP$^+$ cells without direct in situ analysis. Thus, it appears that *RAG* expression in the periphery is found among a population of immature B-cell emigrants from the bone marrow; however, its expression in GC B cells remains questionable.

Somatic hypermutation remains the major mechanism for diversification in the GC reaction. Hypermutation does not solely target the Ig locus, with more evidence for bcl-6 mutation in GC B cells [45,46]. Casali and colleagues have produced a cell line model for hypermutation (CL-01) that displays typical patterns in both Ig [47] and bcl-6 genes [48]. Deficiencies in mismatch repair (Msh2–/– mice) result in destabilized small GC reactions with increased local apoptosis and decreased memory formation [49]. Slight reductions in mutation frequency can be seen using nonselectable transgene targets, suggesting a secondary influence on mutation pattern [50]. DNA polymerase β was recently excluded as the mutator [51], while a DNA polymerase μ has been added as a new candidate [52]. In a similar manner, Honjo and colleagues have cloned an RNA-editing activation-induced cytidine deaminase (AID) that is selectively expressed in GC B-cells and may play a role in somatic hypermutation by acting on RNA intermediates [53••]. Hence, the precise mechanism underlying somatic hypermutation in GC still remains unresolved.

Selection in the GC reaction

Affinity maturation in the GC reaction is achieved through positive selection of centrocytes expressing high-affinity variants of their original antigen receptor. A provocative study by Xu and Davis suggests that the key determinant for selection of antigen-specific B cells before mutation is their CDR3 regions [54•]. Mutation of identical receptors (that vary only in their CDR3) with subsequent selection drives impressive levels of affinity maturation for a variety of antigens in this model. The mechanism that underlies GC selection is still mostly speculation. Complement receptors are known to trap immune complexes on FDC that are then used to 'test' centrocyte affinity for native antigen. The low-affinity FcRγIIB on FDC may also play a role in this selection process by modulating GC Th cell–centrocyte activity[55]. FcRγIIB is also expressed on GC B cells and may regulate apoptosis susceptibility through recruitment of SHIP to the antigen-receptor complex [56]. Other modulators of antigen-receptor signaling may help to integrate the B-cell response to antigen (such as CD19 and CD22)[57]. Bam32, a new adaptor protein that is increased in GC B cells, can also modulate antigen-receptor signaling downstream of phosphatidylinositol 3-kinase (PI-3K) activity and significantly influence the cellular outcome of receptor engagement [58]. Nevertheless, positive selection in the GC may lead to GC cycle reentry for further rounds of diversification and selection, or to exit from the GC reaction into the long-lived memory compartment.

The more likely outcome of random Ig gene hypermutation is decreased affinity for antigen that results in negative selection. The suppressor of apoptosis Bcl-x_L appears to set thresholds for negative selection in the GC with transgenic overexpression permitting a greater variety of lower-affinity variants to enter the memory pool [59]. Telomerase activity also appears critical to the survival of GC B cells and the establishment of long-term memory in a manner that is independent of specificity [60]. There is also evidence that the sympathetic nervous system plays a role in the suppression of GC formation and the development of memory cells in the absence of norepinephrine *in vivo* [61•]. Dying centrocytes in the GC microenvironment are rapidly cleared by resident tingible body macrophages. In general, the primary response GC reaction persists *in situ* for approxi-

mately 21 days after initial priming with some level of affinity maturation continuing by non-GC clonal selection for several weeks [62].

Post-germinal center B cells

The main function of the GC reaction is to produce memory B cells. The memory B-cell compartment can be broadly considered to contain long-lived terminally-differentiated plasma cells and committed (but quiescent) memory B cells that act as precursors to the recall response. The plasma cells are thought to reside mainly in the bone marrow, whereas the memory response precursors recirculate freely to survey for secondary antigen exposure. This latter population is typically regarded as isotype-switched, somatically mutated $B220^+$ B cells. Absence of LT-α disturbs normal splenic architecture and decreases primary B-cell responses. A recent study by Chaplin and colleagues implicates LT-α in the efficient expression of B-cell memory [63]. T-cell memory appears intact, but wild-type memory B-cells were unable to express memory Ig in a LT-α deficient animal.

White and Gray recently demonstrated the presence of secretory IgD only after antigen recall[64•]. These data indicated the presence of IgD^+ memory B cells and a previously unappreciated role for secreted IgD in a memory immune response. There are recent studies on the differential migratory properties of memory B cells able to bind E-selectin (and not P-selectin) through novel sialic acid–containing glycoproteins [65]. Manser and colleagues have also probed the recall response of a dominant anti-hapten clonotype and demonstrate plasma cell development with no induction of mutation [66•]. Their analysis of the secondary GC reaction also suggests the recruitment of memory cells into this GC reaction and the reinitiation of an affinity maturation process.

Our recent analysis of an antigen-specific memory B-cell response revealed a novel B220-negative (using mAb 6B2), non–antibody-secreting memory B-cell compartment [67••]. $B220^+$ memory B cells were also present in this response; however, the $B220^-$ memory B cells rapidly dominate both the recall response and the quiescent memory compartment (assessed 6 weeks postrecall). The vast majority of antigen-binding B cells in the bone marrow also expressed the $B220^-$ phenotype. Upon adoptive transfer, the $B220^-$ memory B cells rapidly self-replenish and differentiate into antibody-secreting cells acting as committed precursors for the memory response. More recently, we demonstrated that these $B220^-$ memory cells emerge during the primary response as a dominant post-GC B-cell compartment (Driver DJ, McHeyzer-Williams L, Cool M, et al.; submitted). These memory B cells re-circulate through spleen and bone marrow for at least 8 weeks after initial priming as memory response precursors. Their cell surface phenotype and location is non-GC and suggests a very different pattern of antigen responsiveness. These studies reveal a more complex cellular organization of the memory B-cell compartment than has been previously appreciated

Acknowledgments

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Our review focuses on B cell memory and concentrates on pertinent 1997/98 studies. Acts as a good precursor to the current review.

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FDC development appears to require signals through LTβR to FDC precursors in the bone marrow. Origins and development of these critical organizers of immune microarchitecture still remains controversial.

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36 Li L, Zhang X, Kovacic S, Long AJ, Bourque K, Wood CR, et al.: Identification of a human follicular dendritic cell molecule that stimulates germinal center B cell growth. J Exp Med 2000, 191: 1077-84

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Murine GC B cells change their expression of an activation antigen BLA-1 over the course of a primary immune response. This antigen may provide an indication of physiological change within the GC B cell compartment.

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42 Yu W, Nagaoka H, Jankovic M, Misulovin Z, Suh H, Rolink A, et al.:
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This model begins the controversy on RAG re-expression in the periphery. These workers use a GFP tagging strategy to follow RAG expression and conclude that RAG is continually expressed by B cells recently emerging from the BM (and not re-expressed as originally reported by Kelsoe and others).

43 Nagaoka H, Gonzalez-Aseguinolaza G, Tsuji M, Nussenzweig MC:
• Immunization and infection change the number of recombination activating gene (RAG)-expressing B cells in the periphery by altering immature lymphocyte production. J Exp Med 2000, 191: 2113-20.

This second study using the GFP-tagging strategy (as in 42 above) identifies a population of immature B cells that migrate to the spleen in response to adjuvant stimulus. These workers see no recruitment of these RAG expressing cells into the GC reaction.

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